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TITLE: The Role of Telomeric Repeat Binding Factor 1 (TRF1) in Telomere Maintenance and as a Potential Prognostic Indicator in Human Breast Cancer

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13. ABSTRACT (Maximum 200 Words)
The aims of this study are to (i) determine the relationships between the telomere binding protein Telomere Repeat Binding Factor 1 (TRF1) and other telomere binding proteins, (ii) establish the potential of TRF1 as a surrogate marker for telomere content (TC) and as a potential clinical marker and (iii) characterize the relationship between of the telomere binding protein TRF1 and TC. Through examining the role of TRF1 in telomere length control and in breast cancer progression, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all specific aims; as outlined in the Statement of Work, are expected to be completed on schedule. The research is in progress.

14. SUBJECT TERMS
Human breast cancer tissue, telomeres, telomere-binding proteins, immunohistochemistry, RT-PCR, slot blot, SIRNA

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I. INTRODUCTION

The aims of this study are to determine the relationship of the telomere binding protein TRF1 to other telomere binding proteins, establish the potential of Telomere Repeat Binding Factor 1 (TRF1) as a surrogate marker for telomere content and as a potential clinical marker and further, to characterize the relationship of the telomere binding protein TRF1 to telomere content. Through examining the role of TRF1 in telomere length and in breast cancer, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, Specific Aim 2 has been completed and a manuscript based on the data generated is in progress. Two of the tasks in Specific Aim 1 were delayed due to a technical issue, however this issue has been overcome and the data collection is in progress and expected to be completed on time. Specific Aim 3 is on schedule.

Hypothesis and Rationale

Telomere Content (TC) has prognostic value in breast cancer; however the factors that control TC are poorly understood. In vitro studies have shown that high levels of TRF1 can stabilize short telomeres and the preliminary results suggest the levels of TRF1 mRNA are related to TC. Together this data suggests that TRF1 level may be directly related to telomere content and therefore be a potential biomarker for telomere length. However, TRF1 also has multiple interacting partners, TRF1 Interacting Nuclear Factor 2 (TIN2), Tankyrase, Telomere Repeat Binding Factor 2 (TRF2) and Protection of Telomeres 1 (POT1), which may modify the interaction between TRF1 and TC. If increases in TRF1 are partially responsible for decreased TC, a prognostic marker of poor outcome, then targeting TRF1 may be a good preventive treatment of breast cancer progression. However, it is also possible that the observed increase in TRF1 is a cellular reaction in response to low TC and therefore a good surrogate for TC. Theses two scenarios must be tested to evaluate the prognostic significance of TRF1 in human breast cancer. Therefore *I hypothesize that defining TRF1 levels using immunohistochemistry could provide a surrogate measure for TC that would be easily adaptable to the clinical setting*. To test this hypothesis I will assess the potential prognostic value of the TRF1 in human breast tumor samples. Additionally, I propose to characterize the relationship of TRF1 to TC, and to TIN2 and Tankyrase to further examine the relationship of TRF1 to TC. I will evaluate this hypothesis through three specific aims.

- **Specific Aim #1**
  Assess the potential of TRF1 protein levels as a surrogate for Telomere DNA Content (TC) in frozen and paraffin embedded breast tumor tissues.

- **Specific Aim #2**

Assess the potential modification of the relationship between TC and TRF1 mRNA levels by TRF1 interacting protein 2 (TIN2) and Tankyrase in frozen human breast tumor samples.

- **Specific Aim #3**
  Examine the effects of increased TRF1 expression on TC and decreased TC on TRF1 expression in breast cancer cell lines.

II. KEY RESEARCH ACCOMPLISHMENTS

IIa. RESEARCH ACCOMPLISHMENTS

- There exists an association between the levels of TRF1, TIN2 and POT1 mRNA within breast tumors, as measured by real-time RT-PCR.

- The levels of TRF1mRNA are not associated with the mRNA levels of the human telomerase reverse transcriptase (hTERT) mRNA or the levels of TRF2 mRNA within breast tumors.

- The levels of TIN2, TRF1, TRF2 and POT1 mRNA are all associated with telomere content.

- Visualized TRF1 and TRF2 distribution by Immunohistochemistry.

- Developed siRNA for hTERT.

- Developed TRF1 overexpression vector.

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the previous annual review, the PhD candidate has had continuing opportunities to work and interact with oncologists, pathologists and other PhD scientists who specialize in breast cancer. These interactions have occurred though tumor board meetings, journal clubs, special seminars and direct interaction within the laboratory. To the training in microscopy she received in the first year of the award, she has received training in cryosectioning and paraffin sectioning by a research technician in the Pathology Department of the School of Medicine and training in flow cytometry by an expert from the UNM Shared Flow Cytometry Resource.

On an educational level, the candidate has written a section and co-instructed the upper-level undergraduate course, Biochemical Laboratory Methods. Additionally, the candidate received training in teaching using the problem based learning model, which she applied as a tutor for the Genetics and Oncology Block at the UNM School of Medicine. The candidate has aspirations of continuing her career in research and remaining in academia and felt teaching provided an opportunity to develop the essential teaching skills need for her chosen career.

IIc. PERFORMANCE ACCOMPLISHMENTS:

Experimental Milestones

Specific Aim 1: (7 tasks)

Task 1 Month 1-2 Completed in year 1

- Purify DNA from paraffin embedded breast tumor samples previously collected by our laboratory.

Task 2 Month 2-6 Completed in year 1

- Measure TC in paraffin embedded breast tumors samples.

Task 3 Month 6-12 Completed in year 1

- Optimize TRF1 antibody for use in frozen tissue and paraffin embedded breast tumor tissue.

  TRF1 antibody specificity has been demonstrated in breast cancer cell-line MCF-7 and conditions for antigen retrieval and staining have been determined.

Task 4 Month 12-14 **In Progress and Modified**

- Section frozen human breast tumor samples and stain with antibody to TRF1.

  Further tests of immunohistochemical staining using an antibody to TRF1 in breast cancer cell lines, has shown a relationship between cell cycle and intensity of TRF1 staining. This makes TRF1 staining a poor clinical stain. The candidate has continued to perform immunohistochemistry staining using TRF1 to answer basic questions about the distribution of TRF1 staining in breast tumors. However, she also decided to develop staining procedures for TRF2 and TIN2 both of which also demonstrated a relationship to Telomere Content and also demonstrate potential as a clinical tool. Staining conditions for TIN2 could not be found however, the staining protocol for TRF2 has been determined and both TRF1 and TRF2 stains are being done in frozen breast tissue. The student hopes to use TRF2 staining to answer previously posed clinical questions regarding the development of a stain related to prognosis, clinical markers and Telomere Content.

Task 5 Month 13-14 **Completed**

- Assess relationship between normalized TRF1 mRNA levels and TRF1 staining intensity.

  Student demonstrated that the levels of TRF1 mRNA as measured by quantitative real-time PCR relate to the staining intensity of TRF1 visualized in various breast cancer cell lines by immunohistochemical staining.

Task 6 Months 14-24 **In Progress**

- Section paraffin embedded breast tumor tissues and stain with antibody to TRF1.

  Paraffin embedded tissues have been sectioned and are currently being stained for both TRF1 and TRF2 using immunohistochemistry.

Task 7 Months 12-30 **Initiated**

- Score sections stained with TRF1 antibody and compare to TC data, histological markers and survival data.

  Data collection has not been completed from the paraffin embedded tissue, however collection is in progress.

Specific Aim 2: (4 tasks) Completed

Task 1 Month 1-2 Completed in year 1

- Extract RNA from frozen breast tumor samples already collected by our laboratory. Design and order Tankyrase and TIN2 primers and probe.

  RNA was extracted from 36 breast tumors. Primers for Tankyrase and TIN2 were designed.

Task 2 Month 2-4 Completed in year 1

- Optimize Tankyrase and TIN2 RT-PCR

  TIN2 RT-PCR was optimized, however Tankyrase primers picked up both Tankyrase 1 and the analog Tankyrase 2. The expression levels of these two proteins are quite different and Tankyrase 2 is highly expressed and functionally not associated with telomere management. Assessment of Tankyrase by RT-PCR yielded an inconclusive result in all experiments. RT-PCR experiments to determine Tankyrase mRNA levels have been placed on hold pending new methods to delineate these two analogs at the molecular level. Recent studies have determined the regulation of Telomere length by proteins to involve a number of complexes, which include TRF1 and TIN2, and also include Tankyrase, POT1 and TRF2. As levels of Tankyrase mRNA could not be determined and POT1 and TRF2 levels may be associated with TRF1 mRNA levels and involved in telomere content determination, RT-PCR reactions were optimized for TRF2 and POT1 as well.

Task 3 Month 4-7 Completed in year 1

- Measure Tankyrase and TIN2 mRNA levels by RT-PCR in RNA extracted from frozen breast cancer samples.

  Tankyrase mRNA levels could not be assessed; however TIN2, POT1 and TRF2 mRNA levels were assessed in 36 frozen breast tumor samples.

Task 4 Month 7-12 Completed in year 1

- Analyze association between Tankyrase and TIN2 mRNA levels with TC and TRF1 mRNA expression.

  Tankyrase mRNA levels could not be assessed so no comparison was possible. TIN2 mRNA levels showed a strong association with TC and TRF1 levels as well as two other telomere binding proteins; POT1 and TRF2.

Specific Aim 3: (6 tasks)

Task 1 Month 12-15 **Completed**

- Design and test small interfering RNAs (siRNA) of human Telomerase Reverse Transcriptase (hTERT)

  The candidate designed several siRNAs against hTERT and tested these siRNAs. The siRNAs showed a reduction in hTERT expression by quantitative real time PCR.

Task 2 Month 15-27 **In Progress**

- Express siRNA of hTERT in breast cancer cell lines and examine TRF1 mRNA levels and TC by RT-PCR and slot blot over time.

  Student is currently following the slow telomere attrition using a slot-blot method of analyzing telomere content. In an effort to speed the loss of telomere, the candidate is currently examining the use of drugs that stabilize the G-quadruplex structures that can be created in the telomere region. The use of these drugs provides rapid telomere attrition.

Task 3 Month 15-18 **Completed**

- Design, generate and test TRF1 expression vector.

  Student has designed and generated a TRF1 expression vector, which demonstrates an increase in TRF1 mRNA levels when examined by quantitative real time PCR.

Task 4 Month 18-30 **In Progress**

- Overexpress TRF1 in breast cancer cell lines and examine TC levels by slot blot over time.

  Data is currently being generated and collected from cells overexpressing TRF1.

Task 5 Month 30-34 **Not Initiated**

- Analyze relationship between TRF1 and TC.

Task 6 Months 30-36 **In Progress**

- Prepare and submit manuscripts.

Candidate is currently preparing a manuscript based on the data generated in Specific Aim 2.

Education and Training Milestones (6 tasks)

Task 1 Month 1-6 Completed in Year 1

- Learn to recognize morphology and features of different types of breast cancer under the guidance of Dr. Nancy Joste.

Student has examined various types of breast cancer and can recognize features of different tumors and tumor stages.

Task 2 Month 1-36 **Continuing**

- Attend tumor board meetings and monthly Cancer Research and Treatment Center Meetings to gain understanding of current treatments for breast cancer and ongoing clinical trials.

Student continues to attend tumor board and the Cancer Research and Treatment Center Meetings on a regular basis and has developed a working relationship with several of the doctors to allow further understanding of current treatments and clinical trials in breast cancer.

Task 3 Month 1-6 **Completed**

- Attend the University of New Mexico School of Medicine medical student training Neoplasia block.

Student attended all the lectures the Genetics and Neoplasia block and also acted as a tutor for the problem based learning sections of the Genetics and Neoplasia block.

Task 4 Month 6-12 **Completed**

- Learn staining procedures and significance of histological markers commonly used in breast cancer under the guidance of Dr. Nancy Joste.

Student has learned basic staining procedures and has gained understanding of the commonly used markers to determine treatment in breast cancer.

Task 5 Month 12-24 **Completed**

- Work with oncologists in the University of New Mexico Hospital to gain perspective on breast cancer.

Student has developed a working relationship with several doctors in the Cancer Center and has used these relationships to develop an understanding of patient care and current issues in breast cancer treatment.

Task 6 Months 12-36 **In Progress**

- Present ongoing work at local and national meetings

III. REPORTABLE OUTCOMES

Presentations:

Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, Philadelphia, June 8-11 2005. "Levels of Telomere Protein mRNAs are Predictive of Telomere Content in Human Breast Tumors." Kimberly S. Butler, William C. Hines, Diana Roberts, Colleen A. Fordyce, Jeffrey K. Griffith (Appendix A)

AACR Special Conference: "The Roles of Telomeres and Telomerase in Cancer" San Francisco, Nov 3-7, 2004. "Levels of Telomere Protein mRNAs are Predictive of Telomere Content in Human Breast Tumors." Kimberly S. Butler, William C. Hines, Diana Roberts, Colleen A. Fordyce, Jeffrey K Griffith.

IV. CONCLUSIONS

To date, all tasks; as outlined in the Statement of Work are on schedule. Two of the tasks in Specific Aim #1 were delayed due to a technical issue, however this issue has been overcome and data collection is in progress and expected to be completed on time. Specific Aim #2 has been completed and a manuscript based on the results from this body of work is in progress. The tasks for Specific Aim #3 have yet been initiated, and are proceeding on schedule. The PhD candidate has completed her educational goals.

Appendix A

LEVELS OF TELOMERE PROTEIN MRNAS ARE PREDICTIVE OF TELOMERE CONTENT IN HUMAN BREAST TUMORS

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Department of Biochemistry and Biophysics, University of California (CAF)

A model of telomere length control has emerged from previous studies in human cell lines, in which telomere-associated proteins act through two mechanisms to control telomere length. In the first mechanism, levels of TRF2 determine the rate of telomere attrition and the minimal telomere length in non-senescent cells. In the second mechanism, the TRF1 complex controls the loading of POT1 onto the telomere, which prevents telomerase from elongating the telomere. Based on this model, we hypothesize that the relative levels of several or all of these telomere-associated proteins are determinants of telomere length in human tumors.

We assessed the relationships between the mRNA levels of TRF1, TRF2, TIN2, POT1 and the telomerase protein component (TERT) to telomere length in 36 human breast tumors. Telomere length was measured as telomere content (TC). Levels of the telomere-associated proteins mRNA were assessed by quantitative RT-PCR. Statistical modeling was performed using SAS software version 8.2 and JMP 5.1.

Linear regression revealed significant, negative, linear associations between TC and the mRNA levels of TRF1, TRF2, TIN2 and POT1 ($p=0.004$; 0.006; 0.003 and 0.042, respectively; $r2=0.22$; 0.20; 0.23; and 0.12, respectively). The significant p-value and weak r2 for each of the individual mRNAs suggest no single factor controls telomere length. Therefore, the General Linear Model regression procedure was used for predicting TC using mRNA levels of the telomere-associated proteins. Using the partial F test, it was determined the mRNA levels of the telomere-associated proteins are all necessary to predict TC ($p<0.0001$). Next, all possible two-way interactions were tested and were determined to be significant ($p=0.0026$, $r2=0.76$). Subsequent analyses indicate that three-way interactions are also important ($p=0.0690$, $r2=0.89$). The increase in r2 resulting from the inclusion of the three-way interactions is significant, supporting the conclusion that the three-way interactions are necessary to predict TC.

To determine which specific interactions are significant in predicting TC, the three-way interaction with the least significant p-value was excluded and the regression model was repeated until only the interactions that were statistically significant at level $p<0.05$ remained. These data demonstrate that TRF1 and TRF2 are not independently associated with TC, nor is the interaction between TRF1 and TRF2. However, the individual

interactions of TRF1 with TIN2, POT1 and TERT (p=0.0103; 0.0129 and 0.0209) and TRF2 with TIN2, POT1 and TERT (p=0.0092; 0.0248 and 0.0119) are each required to predict TC. The separate interactions between TERT and TIN2 and POT1 (p=0.0268 and 0.0350) are also needed to predict TC. The data demonstrate all of the mRNA levels are required to predict TC in human breast tumors.

In summary, the present study provides further support for the complex model of telomere length regulation that was derived from well-characterized human cell lines and suggests that this model is applicable to human breast tumors.